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Prevalence of disease-related DNA polymorphisms among participants in a large cancer prevention trial

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Genetic susceptibility polymorphisms may be of substantial importance in the modulation of cancer risk. The prevalence for an array of polymorphic genes was determined in a cohort of male smokers who participated in a cancer prevention trial in Finland. A random sample of 120 individuals was selected from the trial cohort and the prevalence of variant alleles for nine genes was determined using a polymerase chain reaction-based approach. The prevalence values from this study were also compared with those of other populations derived from previous studies. Our results show that, with the exception of cytochrome P450–1A1 (CYP1A1) and cytochrome P450–2E1 (CYP2E1), all genes tested were sufficiently polymorphic to warrant an investigation of gene-environment studies. Most of the variant alleles, including alcohol dehydrogenase 3 (ADH_3), glutathione-*S*-transferase (GSTM1), methionine synthase (MS), methylene tetrahydofolater reductase (MHTFR), CYP2E1 and CYP1A1, exhibited similar frequencies to other Caucasian populations. Interestingly, the prevalence of androgen receptor-CAG repeat (AR-CAG) and vitamin D receptor (VDR) polymorphisms differed significantly between the alpha-trocopherol, beta-carotene (ATBC) Study and other Caucasian populations. We present herein results from this survey and conclude that the ATBC study population in Finland is sufficiently heterogeneous to facilitate analysis of genetic polymorphisms and disease associations.

Key words: ATBC study, cancer, genotype, polymorphism.

Introduction

The risk of developing cancer is determined by both the number and nature of cumulative carcinogenic exposures and by individual genetic variations. Relevant to the latter, genetic variations in detoxification enzymes, metabolic enzymes and hormone-related genes have been related to cancer risk (Kitson *et al.*, 1995; McWilliams *et al.*, 1995). For example, genetic polymorphisms in detoxification enzymes can substantially alter the metabolic activation and the ultimate elimination of carcinogenic substances. Most pro-carcinogens are first metabolically activated by phase I enzymes (the cytochrome P450s or CYPs) to exert their tumourigenic potential. The activated carcinogens are then detoxified by the phase II enzymes, including glutathione-*S*-transferases (GSTs), *N*-acetyl transferases (NATs), and nicotinamide adenine dinucleotide phosphate (NADP) quinone oxidoreductase (NQO1). Other susceptibility

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gene polymorphisms related to cancer risk include those affecting hormone receptors such as those for oestrogens, androgens, and vitamin A and D (i.e. ER, AR, and VDR), alcohol metabolism (e.g. alcohol dehydrogenase or ADH), and folate and methyl-group metabolism [e.g. methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MS)].

The evaluation of genetic polymorphisms in the context of epidemiological studies can lead to a deeper understanding of the risks resulting from specific exposures. This may be particularly useful when associations are weak or when there is little heterogeneity in exposure level. The assessment of such genetic influences on cancer incidence is one line of inquiry in the Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Study, a large randomized intervention trial conducted in Finland (Albanes *et al.*, 1996). This will complement and enhance ongoing observational analyses within the ATBC Study cohort, including, for example, those of serum vitamin D and folate (Glynn *et al.*, 1996; Tangrea *et al.*, 1997). Our intent is to evaluate genetic polymorphisms as modifiers of cancer risk due to exposures such as tobacco, alcohol, diet, hormone status and intervention assignment (β-carotene and α-tocopherol).

The population of Finland has long been genetically isolated by geography and is considered to be genetically homogeneous. We therefore conducted a prevalence study of several gene polymorphisms to determine whether the ATBC Study population was sufficiently heterogeneous for analyses of gene-disease associations. We were interested also in a multitude of genes because relatively little is known about how various combinations of several genes affect risk. To our knowledge, few, if any, studies have investigated the prevalence of multiple genetic polymorphisms within one population.

Subjects and methods

Study population

The source population consisted of 29 133 white male smokers participating in the ATBC Study conducted in Finland. The ATBC Study was a randomized, placebo-controlled prevention trial designed to determine whether α-tocopherol (50 mg/day), β-carotene (20 mg/day), or both would reduce the incidence of lung, prostate and other cancers. The overall design, rational and objectives of this study have been published (ATBC Cancer Prevention Study Group, 1994) as have the main trial findings and a number of other observational studies (ATBC Cancer Prevention Study Group, 1994; Albanes *et al.*, 1996; Glynn *et al.*, 1996; Tangrea *et al.*, 1997). A random sample of 120 participants (age range: 50–69 years) was selected for deoxyribonucleic acid (DNA) polymorphism prevalence analyses based on the availability of a frozen sample of whole blood obtained (between 4 and 7 years after the start of intervention) from 20,305 men.

DNA isolation

The whole blood samples were lysed in buffer containing 1% Triton X-100TM, 10 mM Tris pH 8.0, 320 mM sucrose and 5 mM $MgCl_2$ and rocked end over end for 30 min. The nuclei were platted by centrifugation at 1000*g* for 5 min. Supernatants were saved and pellets were washed with 2 ml of TNE [25 mM Tris pH 8.0, 150 mM NaCl and 25 mM ethylenediaminetetraacetic acid (EDTA)] and incubated with 3 ml of freshly prepared proteinase-K solution (25 mM Tris pH 8.0, 25 mM EDTA, 500 mM NaCl, and 0.5% sodium dodecyl sulfate (SDS), 400 μg/ml proteinase-K). Samples were incubated overnight at 55°C with end-to-end rocking. The samples were extracted with an equal volume of phenol : chloroform : isoamyl alcohol (25:24:1) followed by a final chloroform extraction step. DNA was precipitated using two volumes of ice-cold ethanol, spooled, and then air-dried. DNA was then resuspended in 200 μl Tris-EDTA (10 mM Tris and 1 mM EDTA, pH 8.2) and stored at −20°C until used.

Genotype analyses

We used a polymerase chain reaction (PCR)-based approach for genotyping. Briefly, PCR fragments were amplified from DNA using specific sense and antisense primers (Table 1). The variant alleles were detected by restriction enzyme digestion and separated by agarose gel electrophoresis and then visualized with ethidium bromide staining. Each of the individual gene polymorphisms were assayed as previously described (Table 1). The GSTM1 assay used did not distinguish between heterozygous (GSTM1*1/0) and homozygous present (GSTM1*1/1) genotypes.

AR receptor trinucleotide CAG repeats analysis was performed by PCR amplification of the CAG repeat region using a ^{32}P-labelled primer (Irvine *et al.*, 1995). After amplification, the PCR product was separated on a sequencing gel and DNA products

Table 1. Primers and restriction enzyme used for genotyping assays

Locus	Sense primer	Antisense primer	Diagnostic enzyme	Ref.
CYP1A1	5′-CCA GGA AGA GAA AGA CCT TCC AGC GGG CCA-3′	5′-GAA CTG CCA CTT CAG CTG TCT-3′	*Nco*1	Ambrosone *et al.* (1995)
CYP2E1	5′-CCA GTC GAG TCT ACA TTG TCA-3′	5′-TTC ATT CTG TCT TCT AAC TGG-3′	*Pst*1	Kato *et al.* (1992)
GSTM1	5′-CGC CAT CTT GTG CTA CAT TGC CCG-3′	5′-TTC TGG ATT GTA GCA GAT CA-3′	–	Zhong *et al.* (1993)
GSTM4	5′-CGC CAT CTT GTG CTA CAT TGC CCG-3′	5′-ATC TTC TCC TCT TCT GTC TC-3′	–	Zhong *et al.* (1993)
AR (CAG repeat)	5′-CCA GAA TCT GTT CCA GAG CGT GC-3′	5′-GCT GTG AAG GTT GCT GTT CCT CAT-3′	–	Irvine *et al.* (1995)
NQO1	5′- TCC TCA GAG TGG CAT TCT GC-3′	5′-TCT CCT CAT CCT GTA CCT CT-3′	*Hinf*1	Kelsey *et al.* (1997)
VDR	5′-CAG AGC ATG GAC AGG GAG CAA-3′	5′-GCA ACT CCT CAT GGC TGA GGT CTC-3′	*Taq*1	Taylor *et al.* (1996)
ADH_3	5′-GCT TAA AGA GTA AAT ATT CTG TCC CC-3′	5′-AAT CTA CCT CTT TCC GAA GC-3′	*Ssp*1	Bosron and Li (1986)
MTHFR	5′-TGA AGG AGA AGG TGT CTG CGG GA-3′	5′-AGG ACG GTG CGG TCA GAG TG-3′	*Hinf*1	Frosst *et al.* (1995)
MS	5′-GAA CTA GAA GAC AGA AAT TCT CTA-3′	5′-CAT GGA AGA ATA TGA AGA TAT TAG AC-3′	*Hae*III	Van der Put *et al.* (1997)

were visualized by autoradiography. Size estimates of the CAG repeats were determined by using known CAG repeat fragments. Due to the difficulty of this assay, we were only able to genotype successfully 82 samples.

Genotyping results were reviewed independently by two investigative groups. Restriction enzyme cutting controls and negative controls (PCR reagents without DNA) were included with each batch.

Statistical analyses

The chi-square test for heterogeneity was used to determine statistically significant differences in allele prevalence using the statistical program, STATA (STATA Corporation, TX, USA).

Results

The prevalence of the activation/detoxification enzymes and other receptor/metabolism polymorphisms are shown in Table 2. The CYP1A1 Ile to Val variant, the CYP2E1 *Pst*1 5′-region polymorphism and the GSTM1 null allele were found in 7%, 2% and 40% of the study population, respectively. The prevalence of the ADH_3 variant allele was 56%. The prevalence of the MTHFR Ala to Val substitution variant allele was 23% and the MS Asp to Gly substitution variant was 17%. The prevalence of the VDR variant allele was 69% and the AR-CAG repeat polymorphisms were 61% for repeats of 22 or more, 39% for repeats of 19–21, and no repeats of 18 or less were found in this population.

Previously reported prevalence of these susceptibility polymorphisms were obtained for control subjects from published observational studies and are shown in Table 3 in comparison to those observed in our study. Genotype frequencies we found were generally comparable to those reported from other predominantly Caucasian populations, with the exception of the AR-CAG repeats and VDR polymorphisms. Frequencies for these genotypes were significantly different compared to the prevalence in other Caucasian populations ($P < 0.01$ for AR-CAG repeats and $P = 0.04$ for VDR).

Discussion

In this study, as a prelude to conducting nested prospective case–control studies, we assessed the prevalence of an array of genes believed to alter people's susceptibility to the development of cancer. To our knowledge, this is the first time such a prevalence analysis of one large study population has been conducted. Our results show that in the ATBC Study participants, all of the genes tested were sufficiently variable with the exception of the distribution of CYP1A1 and CYP2E1, for which 93% and 98% of our sample, respectively, exhibited the wild type (similar to other non-Asian populations). Furthermore, the majority of the polymorphisms in the ATBC Study sample demonstrated

Table 2. Prevalence of selected gene polymorphisms in the ATBC population

Gene	Frequency allele 1	Frequency allele 2	Frequency allele 3
CYP1A1	Ile*	Val*	
	0.93	0.07	
CYP2E1	5′-promoter wt**	5′-promoter variant**	
	0.98	0.02	
	GSTM1 1/1***	GSTM1 0/0***	
GSTM1	0.60	0.40	
	Pro†	Ser†	
NQO1	0.80	0.20	
	CAG ≥22††	CAG19-21††	CAG ≤18††
AR-CAG	0.61	0.39	0
	T‡	t‡	
VDR	0.31	0.69	
	ADH_3-1‡‡	ADH_3-2‡‡	
ADH_3	0.44	0.56	
	Ala§	Val§	
MHTFR	0.77	0.23	
	Asp¶	Gly¶	
MS	0.83	0.17	

*Isoleucine to valine substitution associated with increased activity.
**Variant promoter is linked with higher expression and thus increased risk.
***Absence of (null) of allele linked with increased risk.
†Proline to serine substitution linked to loss of enzyme activity.
††Number of repeats inversely associated with risk.
‡Variant allele (t) associated with decreased risk.
‡‡ADH_3-2 has lower enzyme activity.
§Alanine to valine substitution linked with decreased enzyme activity.
¶Aspartic acid to glycine substitution with unknown functional significance.

prevalences very similar to other Caucasian populations. The GSTM1 and MTHFR type distributions were slightly different, while the AR-CAG repeats and VDR varied significantly, although this could be due to our sample size. In general, our findings add to previous research suggesting that the distribution of polymorphisms does not vary greatly between Caucasian populations. It is interesting to note that the VDR and AR-CAG high-risk alleles for prostate cancer are under-represented in the ATBC study population, and prostate cancer rates in Finland are low compared to the USA (Finish Cancer Registry, 1994). How these may be related needs to be explored.

More importantly from the standpoint of pursuing strategies aimed at cancer prevention, these results demonstrate substantial genetic variability in susceptibility polymorphisms even within a relatively homogeneous population such as the ATBC Study participants in Finland. Modulation of risk derived from exposure to endogenous or exogenous risk factors by these and other genotype/phenotypes may be of substantial importance in human carcinogenesis. This potential population impact derives from the high relative prevalence of polymorphisms such as those coding for the detoxification enzymes. By contrast, genes such as BRCA1 are known to have a low prevalence but a high level of penetrance, with greater than 85% of carriers eventually developing disease (Breast Cancer Linkage Consortium, 1998).

Susceptibility polymorphisms can be investigated with relative ease in studies such as ours in which exposures (e.g. cigarette smoking, diet, serum nutrients, vitamin supplement intervention) that are sources of risk or prevention have been well documented. By controlling for these exposures, risk attributable to the genotype variants is more easily discerned, thereby contributing to our understanding of genetic susceptibility and disease. For example, although heavy smoking is a well-documented and overwhelming risk factor for lung and other cancers, detrimental metabolizing enzyme phenotypes may raise the risk of light and moderate smokers, and the balance between phase I and phase II enzymes may determine the actual dose of carcinogens, and therefore influence cancer risk. This was recently demonstrated in the study by El-Zein and colleagues that showed increased relative risk for lung cancer associated with different combinations of detrimental genotypes (El-Zein *et al.*, 1997).

The information contained in this report is encouraging for the conduct of multiple nested

Table 3. Prevalence of gene polymorphisms in the ATBC Study in comparison to other populations

Gene	Population	Number	Frequency allele 1*	Frequency allele 2	Frequency allele 3	Reference
CYP1A1	ATBC	115	0.93	0.07		This study
	Finnish	242	0.95	0.05		Hirvonen *et al.* (1992)
	Caucasian	228	0.92	0.08		Ambrosone *et al.* (1995)
	Japanese	132	0.79	0.21		Morita *et al.* (1997)
CYP2E1	ATBC	96	0.98	0.02		This study
	Finnish	242	0.99	0.01		Hirvonen *et al.* (1993)
	Caucasians	107	0.98	0.02		Kato *et al.* (1992)
	African-Americans	87	0.95	0.05		Kato *et al.* (1992)
	Japanese	49	0.76	0.24		Kato *et al.* (1992)
	Japanese	132	0.80	0.20		Morita *et al.* (1997)
GSTM1	ATBC	111	0.60	0.40		This study
	Finnish	294	0.47	0.53		Saarikoski *et al.* (1998)
	Am-Caucasians	465	0.48	0.52		London *et al.* (1995)
	Am-Caucasians	233	0.50	0.50		Ambrosone *et al.* (1995)
	African-American	251	0.73	0.37		London *et al.* (1995)
	African-American	132	0.77	0.23		Kelsey *et al.* (1997)
	Mexican-Americans	146	0.60	0.40		Kelsey *et al.* (1997)
	Japanese	132	0.58	0.42		Morita *et al.* (1997)
NQO1†	ATBC	149	0.80	0.20		This study
	Caucasians	114	0.75	0.25		Kelsey *et al.* (1997)
	African-American	136	0.78	0.22		Kelsey *et al.* (1997)
	Asians	118	0.56	0.44		Kelsey *et al.* (1997)
AR-CAG	ATBC	82	0.61	0.39	0.00‡	This study
	Caucasians	588	0.52	0.39	0.09	Giovannucci *et al.* (1997)
	Caucasians	39	0.39	0.43	0.18	Irvine *et al.* (1995)
	African-American	44	0.25	0.27	0.48	Irvine *et al.* (1995)
	Asian	39	0.51	0.39	0.10	Irvine *et al.* (1995)
VDR	ATBC	116	0.69	0.31‡		This study
	Caucasians	162	0.55	0.45		Taylor *et al.* (1996)
ADH_3	ATBC	116	0.44	0.56		This study
	Americans, white	–	0.50	0.50		Bosron and Li (1986)
	Americans, black	–	0.85	0.15		Bosron and Li (1986)
	Japanese	–	0.95	0.05		Bosron and Li (1986)
	Puerto Ricans	146	0.62	0.38		Harty *et al.* (1997)
MHTFR	ATBC	101	0.77	0.23		This study
	Caucasians–PHS	326	0.65	0.35		Ma *et al.* (1997)
	Caucasians	627	0.66	0.34		Chen *et al.* (1996)
MS	ATBC	112	0.83	0.17		This study
	Dutch	364	0.84	0.16		Van der Put *et al.* (1997)

*Designation of individual alleles specified in Table 2.
†NQO1 sample from different individuals in the same ATBC population.
‡Chi square test comparing ATBC to other Caucasians $P \leq 0.05$.

case–control studies designed to explore associations between these and other susceptibility polymorphisms and various cancers within the ATBC Cancer Prevention Study. In addition, this study is unique due to prospective case ascertainment among individuals blindly randomized to placebo or vitamin intervention. To our knowledge this is one of the few studies in which cancer risk, vitamin supplementation and genotype can be explored mechanistically. The importance of studying chemopreventive agents in the context of gene-environment interactions has been demonstrated previously. For example, Probst-Hensch and group (Probst-Hensch *et al.*, 1998) showed that the absence of the GSTM1 gene increases cytochrome P4501A2 activity among consumers of cruciferous vegetables, suggesting that chemopreventive agents and foods can modify the effects of genotype.

Future investigations will include both single and joint risk effects of multiple polymorphism combinations and will assess interactions between susceptibility genes and other endogenous (e.g. hormones) and exogenous (e.g. tobacco smoke, vitamin supplementation) risk factors. To afford appropriate and rigorous hypothesis testing, these studies will require reasonable prevalence of the relevant alleles, and the present results confirm that this is the case. The congruence observed with other studies reporting population prevalence also supports the generalizability of future findings from this study regarding

these susceptibility factors. We must note, however, that the representativeness of this population may still be suspect as it is comprised of older male smokers selected for a clinical trial. Furthermore, the whole blood samples were obtained after 4–7 years of participation in the intervention trial. If susceptibility genotypes are associated with survival and/or intervention, this could introduce a selection bias into our study cohort. In addition, the prevalence estimates in the current study may be limited by the small sample size. However, here we report that genotype prevalence estimates in the ATBC Study are concordant with estimates in other Caucasian populations.

In conclusion, appraisal of multiple genotypes is becoming increasingly more important in cancer risk assessment. As our knowledge of carcinogenesis and associated genes expands, so does the need for more complex studies exploring gene-to-gene and gene-to-environment interactions. We show here that this population sample of Finland is sufficiently genetically heterogeneous to permit genotype-based cancer risk assessments. Furthermore, the ATBC Study population provides us with the ability to conduct nested case–control studies to explore associations between multiple polymorphisms and cancer risk with adequate sample size, and detailed information concerning many relevant factors including diet, anthropometry, smoking and serum biochemistry.

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